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Morphological and Biochemical Studies on Yolk Degradation in the Sea Urchin, *Hemicentrotus pulcherrimus*

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ABSTRACT—The yolk granules of sea urchin, *Hemicentrotus pulcherrimus*, were observed by an electron microscope and classified into 4 types; dense, intermediate, sparse and lysosomal yolk granules, on the basis of their structural characteristics. In unfertilized eggs, most of the yolk granules were dense granules, whereas they were not observed in gastrulae. During the development of the sea urchin embryo, the dense granules decreased, while the sparse granules increased. The yolk granules seem to change from the dense state to the sparse state via the intermediate state. These morphological changes are in accordance with those observed in the yolk granules, which are isolated and incubated in acidic conditions [22].

Some biochemical changes, which underlie these morphological changes, were also investigated. Analyses of yolk proteins by column chromatography and SDS-polyacrylamide gel electrophoresis revealed that overall physico-chemical properties of yolk glycoprotein complex were maintained after proteolytic processing. The lipid composition of the yolk granules was analyzed. In acidic conditions, although triacylglycerol, phosphatidylcholine and phosphatidylethanolamine were lost more than 50%, 70% of cholesterol was retained after the incubation in acidic conditions for 24 hr, when the yolk granules became the sparse state.

INTRODUCTION

The yolk granules are generally considered as storage organelles containing raw materials and energy source to be consumed during the embryogenesis. The yolk granules of sea urchin contain a glycoprotein of apparent molecular weight around 180 kDa, called vitellogen, a major yolk glycoprotein or a major yolk protein [2, 9, 22]. A similar glycoprotein has also been identified in the sand dollar, *Dendraster excentricus* [16]. A precursor to this protein, vitellogenin is synthesized in the intestine of female and male and also in ovaries and testes in *Strongylocentrotus purpuratus* [21]. It has been also reported that vitellogenin is synthesized by coelomocytes and secreted into coelomic fluid [8]. Vitellogenin is processed to vitellogen after incorporation into ovaries and it is accumulated in the eggs. Vitellogen disappears during the development of sea urchin embryos, while lower molecular weight proteins appear in developed embryos such as blastulae and gastrulae [2, 9, 20, 22]. In several species of sea urchins, leupeptin-inhibitable thiol protease(s) play a key role in these changes in composition of yolk proteins [19, 22]. Although vitellogen disappears as development proceeds, remarkable changes in the number and mass of the yolk granules are not observed [2, 12]. Yolk granules of *Hemicentrotus pulcherrimus* have a membrane-limited vescular structure in which electron-dense subparticles of 40–80 nm in diameter are packed. Density of subparticles decreased distinctively in the yolk granules incubated in acidic conditions [22] as observed in gastrulae. Experiments on

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proteolytic processing of vitellogen by incubation of isolated yolk granules in acidic conditions [19, 22] were conducted on the basis of the hypothesis that cleavage of yolk proteins is generated by acidification of yolk granules. In addition, recently, direct evidence indicating that proteolysis of vitellogen is regulated by acidification of yolk granules in sea urchin embryos was reported [13]. Although the mechanism for acidification of yolk granules after fertilization is still unclear, the acidification is to influence not only the composition of yolk proteins but also the composition of other constituents.

In this work, the morphological change in yolk granules during early development of sea urchin, *H. pulcherrimus,* are studied by electron microscopy. Biochemical analyses of compositional changes in constituents of the yolk granules were also performed. The relationship between morphological observations and biochemical analyses is discussed.

MATERIALS AND METHODS

Eggs and spermatozoa of *Hemicentrotus pulcherrimus* were collected by pouring isotonic KCl solution into the opened body cavity. The eggs were fertilized by addition of suitably diluted sperm suspension, and the embryos were cultured at 20°C under continuous stirring of 60 rpm with a synchronous motor. Unfertilized eggs, 16-cell stage embryos, swimming blastulae just after hatching and gastrulae (23 hr after insemination) were fixed for 30 min at 4°C. The fixative contained 1% glutaraldehyde and 1% OsO_4 in artificial sea water buffered with 0.1 M sodium cacodylate (pH 7.0). The fixed specimens were rinsed with the same buffer containing 0.5% EDTA and dehydrated with ethanol series. They were then mixed evenly and stained *en bloc* with lead citrate and uranium acetate. After being embedded in Poly/Bed 812 (Polyscience Inc. Warrington, PA. USA) on a flat plate, embryos having suitable direction were selected. Thin sections (approximately 120 nm) were examined with a Hitachi H300 electron microscope. Classification of yolk granules according to their morphological characteristics and the measurement of their size were carried out on printed images. For more accurate comparison of density of each yolk granule, an image analyzing system was employed. The direct images on a JEM-2000FX electron microscope were put in Excell-II image analyzer (Nippon Avionics Co. Tokyo, Japan) through a CCD camera and processed. The relative brightness of each yolk granule was calculated mechanically. For the reduction of deviation caused by sample preparation, all measurements were performed on a single section.

The yolk granules from unfertilized eggs were isolated and incubated in artificial sea water acidified with citric acid as reported previously [22]. SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli [10]. Protein concentration was determined by the method of Bradford [4] using bovine serum albumin as the standard.

Analysis of lipids was performed as described by Mita and Ueta [14]. Total lipids extracted from yolk granules by the method of Bligh and Dyer [3] were analyzed by high performance thin layer chromatography (HPTLC). HPTLC plates were developed for polar lipids with chloroform-methanol-acetic acid-formic acid-water (35:15:6:2:1) and then for non-polar lipids with hexane-diethylether-acetic acid (70:30:1), according to Macala *et al.* [11]. A standard lipid mixture was applied on each plate. After development, the plates were soaked into cupric acetate (3%)-phosphoric acid (8%) solution and heated at 180°C for 15 min [11]. In most cases, the plates were scanned for densitometry immediately after heating. The amounts of triacylglycerol, phosphatidylcholine, phosphatidylethanolamine and cholesterol were determined from the standard curves of the respective authentic lipids. For analysis of fatty acid composition, isolated triacylglycerol, phosphatidylcholine and phosphatidylethanolamine on a thin-layer chromatography (TLC) plate were subjected to methanolysis by heating with 5% HCl in methanol at 85°C for 2 hr. The fatty acid methylesters were extracted with n-hexane and analyzed by gas-liquid chromatography using a GC-R1A gas-liquid chromatograph (Shimadzu Instrument Co., Kyoto, Japan) equipped with a coiled column packed with 15% EGSS-X (Gasu-

kuro Kogyo Inc., Tokyo, Japan).

The phospholipids, triacylglyceride, cholesterol, cholesterol ester and fatty acid standards were obtained from Sigma (St. Louis, Mo. USA), HPTLC plates (Silica gel 60) and TLC plates (Silica gel 60) were purchased from Merck (Darmstadt, Germany). Ultrogel AcA 34 was from LKB (Upsala, Sweden). DE 32 was a product of Whatman (Maidstone, England). All reagents and solvents used were at the highest grade commercially available.

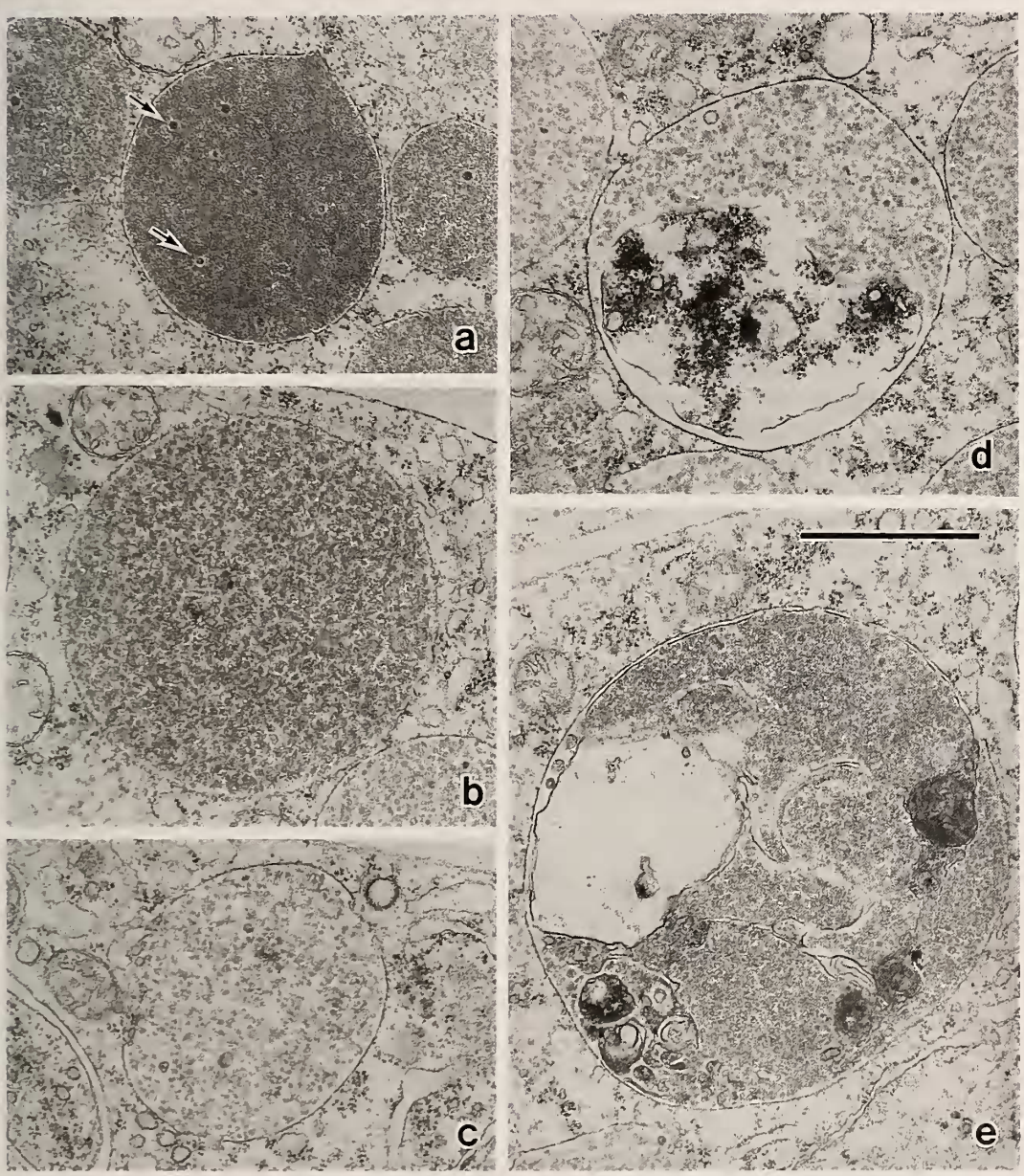

FIG. 1. Yolk granules having different morphological features. Typical "dense" type a), "intermediate" type b), "sparse" type c) yolk granules, and two "lysosomal" type d, e) yolk granules. Each type corresponds to these in Table 1. Note small membrane-bounded particles (arrows). Bar indicates 1 μm, common to a-e).

RESULTS

Morphological observation

Morphological changes in yolk granules during early development were examined with transmission electron microscopy. Unfertilized eggs, 16-cell stage embryos, swimming blastulae and gastrulae were used as developmental stages. In order to minimize differences caused by preparation of samples, similarly fixed specimens were evenly mixed, block-stained, embedded in a flat plate and sectioned in the same thickness. According to their morphological appearance, we classified yolk granules into 4 categories: dense, intermediate, sparse and lysosomal. The first 3 categories were based on the density of yolk particles in each yolk granule. We chose two certain densities, high and low of intermediates, as standards. Yolk granules having higher density than high standard were classified as dense. The granules which have the density between two standard densities and lower density than low standard were classified as intermediate and sparse, respectively. The lysosomal yolk granules were those which fused with or were engulfed into a lysosome. Typical 4 types of yolk granules are shown in Fig. 1. The dense type (Fig. 1a) is limited by a single membrane in which small and electron dense particles were tightly packed. Some of these particles seem to be encircled by a membraneous structure (arrows). The density of particles are moderate in the intermediate type (Fig. 1b), and low in the sparse type (Fig. 1c). Some of the lysosomal type have fused with a lysosome and formed a secondary lysosome (Fig. 1d). Another lysosomal type has been ingested in clumps in a large lysosome. The yolk granules ingested in the secondary lysosome varies in their density. Most of them have an intact limiting membrane (Fig. 1e). This type seems to be formed by the process through autophagic movement. Yolk granules at each developmental stage were classified into these 4 types in electron micrographs. The constitutional rate at each stage is shown in Table 1. The dense type is abundant in unfertilized eggs, while the sparse type is dominant in the gastrula stage. These data indicate morphological transition of yolk granules from the dense type to the sparse type through the intermediate type, in the course of early development. The consitutional ratio of these three types did not remarkably differ from portion to portion in a

Table 1. Changes in the constitutional ratio of yolk granule type of the developing sea urchin embryos, *H. pulcherrimus*

Stage	Characteristics of yolk granules			
	Dense (%)	Intermediate (%)	Sparse (%)	Lysosomal (%)
Unfertilized egg	94	6	0	0
16-cell stage				
Micromere	29	64	7	0
Mesomere	38	62	0	0
Macromere	29	67	4	0
Blastula				
Animal area	2	85	11	2
Vegetal area	3	81	16	0
Mesenchyme	0	79	20	1
Gastrula				
Animal area	0	65	35	0
Vegetal area	0	58	40	2
Archenteron	0	63	35	2
Mesenchyme	0	59	34	7

developmental stage, such as micromere, mesomere and macromere in the 16-cell stage. The ratio of the lysosomal type is very small even at the gastrula stage. In order to confirm the morphological change in yolk granules of developing embryos described above, we employed another method as mentioned in Materials and Methods. For getting more accurate estimation of the morphological changes in yolk granules, the brightness of each granule was measured using the image analyzing system. The statistical data shown in Table 2 indicate that the electron density of yolk granules decreases during embryogenesis. These data accord well with the morphological changes shown in Table 1, although the lysosomal type is eliminated from automated measurement. There is a possibility that expanded volume of yolk granules due to swelling might affect superficial increase of brightness. The total amount of materials included in a granule must be parallel to $1/B \times V$ (where B is relative brightness and V is relative volume). Therefore we measured the diameter of yolk granules on electron micrographs for getting the average diameter. Fifty yolk granules with sharp limiting membrane were randomly selected in each stage. The volume ratio of a yolk granules in unfertilized eggs to that in gastrulae was calculated to be 1 to 1.3. The volume of yolk granules in developing embryos increases. When this value and the relative brightness in Table 2 were applied to the equation mentioned above, the ratio of average sized yolk granules in these two developmental stages was calculated to be 15 to 13. These results show that increase of relative brightness (decrease of electron density) in yolk granules during the development results also from the decrease in the total amount of substances contained in yolk granules due to hydrolysis.

Table 2. Relative brightness of yolk granules during development of embryos

Stage	Maximum	Minimum	Mean ± s.d.	
Unfertilized egg	8.59	4.73	6.70	0.98
16-cell stage	9.54	6.38	7.68	0.77
Blastula	11.22	8.44	9.77	0.80
Gastrula				
Ectoderm	11.03	8.54	9.87	0.60
Endoderm	11.09	8.53	10.03	0.61

Fifty yolk granules were examined.

Biochemical analyses

Vitellogen and its proteolytically processed products, which constitute yolk protein complexes, were purified from unfertilized eggs and blastulae and analyzed as follows. Unfertilized eggs and blastulae were homogonized in 5 volumes of 0.02 M Tris-HCl buffer, pH 7.4, 0.1 M NaCl, 0.1 mM phenylmethylsulfonylfluoride and 0.1 mM N-α-*p*-tosyl-L-lysine chloromethylketone hydrochloride with a Waring blender at 10,000 rpm for 5 min. The homogenate received 1/4 volume of n-butanol and was further homogenized for 5 min. The emulsified homogenate was centrifuged at 10,000 g for 30 min. The centrifugation separated emulsion into 3 phases. The middle aqueous phase was collected as a crude yolk protein preparation and passed through a column of Ultrogel AcA 34 (3× 75 cm) equilibrated with 0.02 M Tris-HCl, pH 7.4, and 0.1 M NaCl at a flow rate of 15 ml/hr (Figs. 2 and 3). Proteins in fractions (10 ml each) eluted from a gel filtration column were analyzed by

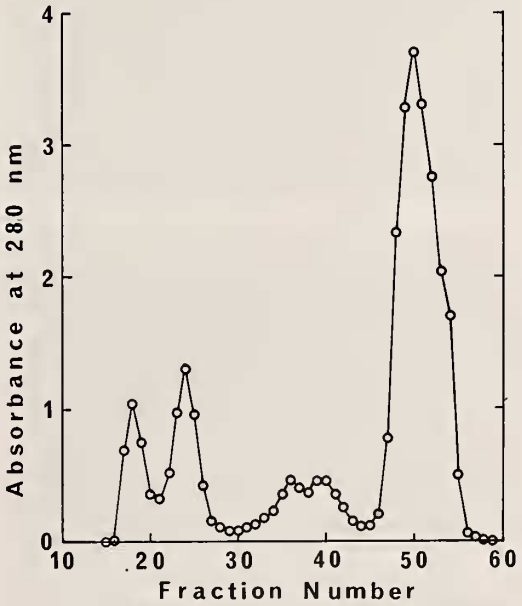

Fig. 2. Gel filtraion of butanol extract from unfertilized eggs on the column of Ultrogel AcA 34. Details in the text.

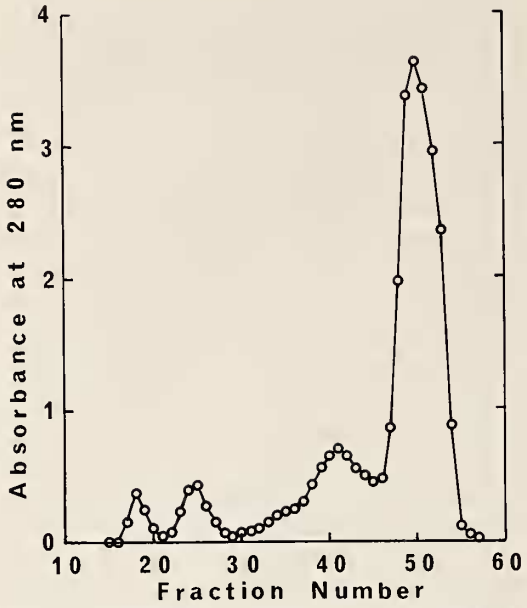

Fig. 3. Gel filtration of butanol extract from blastulae on the column of Ultrogel AcA 34. Details in the text.

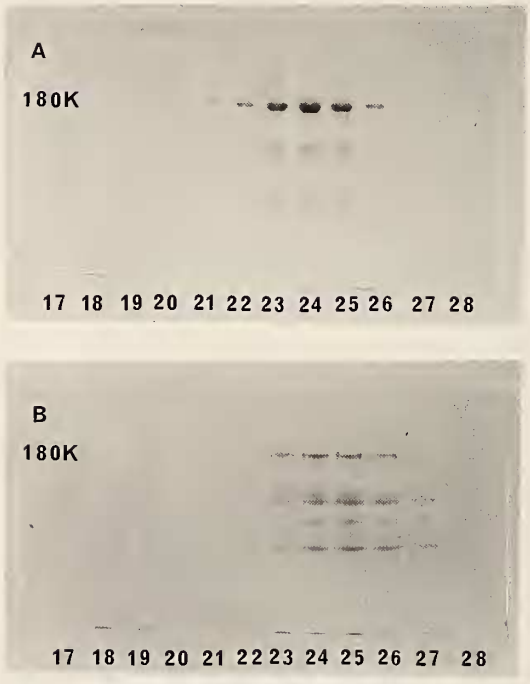

Fig. 4. SDS-polyacrylamide gel electrophoresis of proteins eluted from the Ultrogel AcA 34 column. A, unfertilized eggs. B, blastulae. Numbers under the lanes are numbers of fractions. See details in the text.

SDS-polyacrylamide gel electrophoresis (Fig. 4). Although the elution pattern from the 15th to the 30th fraction observed by absorbance at 280 nm was essentially the same in the unfertilized eggs and blastulae, electrophoregram in unfertilized eggs were different from that in blastulae. The fractions rich in vitellogen in the unfertilized eggs were saved. In blastulae, the fractions of same elution volume as vitellogen of unfertilized eggs were pooled. The collected gel filtrates of unfertilized eggs and blastulae were dialyzed against 0.02 M Tris-HCl fuffer, pH 7.4, and applied onto a DEAE cellulose column (1.4×10 cm) equilibrated with the buffer used for dialysis. The column was washed with the buffer described above. Proteins were then eluted with a linear gradient of NaCl from 0 to 0.5 M in the same buffer (Fig. 5). The protein from both sources eluted from a DEAE cellulose column was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 5). The yolk protein complexes from unfertilized eggs and blastulae, which are composed of vitellogen in unfertilized eggs and of vitellogen-derivatives and vitellogen in blastulae, were eluted with same elution volume in gel filatration and in the same condition on column chromatography. However the electrophoretic pattern of yolk proteins in unfertilized eggs was different from that in blastulae as shown in Figs. 4 and 5. A protein of molecular weight, 180 kDa, (vitellogen) was the most abundant protein in unfertilized eggs, whereas lower molecular weight proteins were predominant in blastulae. The same results were also obtained from gel filtration, DEAE-cellulose column chromatography and SDS-polyacrylamide gel electrohporesis of proteins in yolk granules incubated in isotonic salt solution buffered at pH 4.5 (data not shown), as those in blastulae. These data indicate that overall molecular mass and ionic properties of the yolk protein complexes composed of vitellogen and its derivatives are maintained at least up to the blastula stage, although proteolytic processing of the protein occurs. The application of butanol-extraction suggests that lipid moieties and carbohydrate moieties may not contribute to maintain the form of the yolk protein complex in sea urchin eggs and embryos.

In order to examine whether acidification in yolk

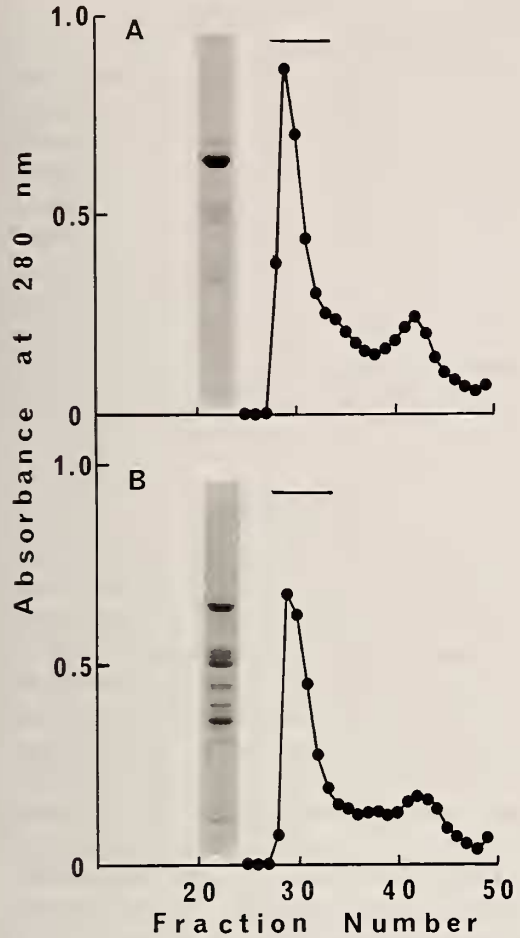

FIG. 5. Column chromatography on DEAE-cellulose of yolk proteins from unfertilized eggs and blastulae, and SDS-polyacrylamide gel electrophoresis of yolk proteins eluted from a DEAE-celluose column. Fractions under the bar were collected and subjected to SDS-polyacrylamide gel electrophoresis. A, unfertilized eggs. B, blastulae, See details in the text.

granules stimulates the hydrolysis of lipids, isolated yolk granules from unfertilized eggs were incubated in isotonic salt solution buffered at pH 4.5, for 12 and 24 hr and lipids in yolk granules were analyzed (Table 3). Changes of protein contents in yolk granules after incubation in acidic conditions are also given in Table 3. Amounts of lipids and protein are expressed in the ratio of remaining amount to the initial amount in the preparation used for the incubation. The experiments were performed in 3 preparations and quantitative values were obtained in duplicate determinations. Although triacylglycerol, phosphatidylethanolamine and phsophatidylcholine in yolk granules were hydrolyzed more than 50% after the incubation for 24 hr, approximately 70% of cholesterol was retained. The fatty acid composition of triacylglycerol, phosphatidylcholine and

TABLE 3. Changes in contents of lipids and protein in isolated yolk granules during incubation in acidic conditions

Constituent	0	12	24 hr
Triacylglycerol	100 (8.0 mg)	50.0	31.2
Phosphatidyl-choline	100 (1.8 mg)	61.1	50.0
Physphatidyl-ethanolamine	100 (0.5 mg)	60.0	40.0
Cholesterol	100 (1.3 mg)	84.6	69.2
Protein	100 (62 mg)	69.4	54.9

The amount in parentheses is the initial amount of each constituent.

TABLE 4. Fatty acid composition of triacylglycerol in the yolk granules incubated in acidic conditions

Fatty acid (%)	0	12	24 hr
14:0	12.5±0.2	12.7±0.2	12.4±0.5
16:0	24.7±1.0	25.1±0.8	26.5±1.1
16:1	4.7±0.6	4.8±0.8	5.0±0.9
18:0	2.2±0.2	2.6±0.5	2.6±0.7
18:1	8.1±0.1	5.5±0.7	5.7±1.0
18:2	1.4±0.2	1.4±0.3	0.9±0.3
18:3	2.8±0.8	2.6±0.6	2.8±0.8
18:4	4.8±1.5	4.8±1.8	4.7±1.2
20:1	11.2±0.4	12.2±0.3	12.3±0.5
20:2	9.6±1.3	9.7±1.5	9.9±1.2
20:3	1.8±0.2	2.2±0.7	1.6±0.3
20:4(n−6)	7.1±0.4	6.9±1.5	7.2±0.8
20:5(n−3)	8.7±0.2	8.5±0.3	8.4±1.0
22:6	1.0±0.2	1.0±0.1	tr.
Saturated acid	38.9±1.3	40.4±1.5	41.5±2.0
Monoenoic acid	24.0±0.5	22.5±0.9	13.0±1.0
Polyenoic acid	37.2±1.0	37.1±1.2	35.5±1.8

The ratio of fatty acids is expressed in mean±s.d.. tr., trace (<0.1%).

Table 5. Fatty acid composition of phosphatidylcholine in the yolk granules incubated in acidic conditions

Fatty acid (%)	0	12	24 hr
14:0	3.0±0.4	2.2±0.8	1.2±0.6
16:0	5.6±0.6	7.1±0.7	4.8±1.0
18:0	3.9±1.0	4.9±1.0	4.6±1.3
18:1	3.3±1.0	2.7±0.6	2.7±1.2
18:2	0.3±0.1	0.5±0.3	0.3±0.2
18:3	0.3±0.1	0.4±0.2	0.4±0.3
20:1	15.3±0.2	19.2±0.3	19.6±1.0
20:2	10.2±0.3	8.9±1.0	8.8±1.2
20:3	tr.	tr.	tr.
20:4(n−6)	27.7±1.0	27.2±0.7	27.0±0.5
20:5(n−3)	30.4±0.7	26.9±0.5	30.6±1.5
Saturated acid	12.5±1.5	14.2±1.2	10.6±2.6
Monoenoic acid	18.6±1.2	21.9±1.3	22.3±1.2
Polyenoic acid	68.9±0.5	63.9±1.0	67.1±2.5

The constitutional ratio is given in mean±s.d.. tr., trace (<0.1%).

Table 6. Fatty acid composition of phosphatidylethanolamine in the yolk granules incubated acidic conditions

Fatty acid (%)	0	12	24 hr
14:0	1.1±0.5	1.1±0.5	1.2±0.6
16:0	11.4±1.0	11.6±1.2	11.3±1.2
18:0	11.0±1.2	12.3±0.9	14.4±2.2
18:1	0.4±0.2	1.9±0.9	3.0±1.5
18:2	0.5±0.3	0.9±0.5	1.5±0.9
18:3	1.5±0.5	tr.	0.2±0.1
20:1	7.3±1.0	7.4±0.8	6.7±0.8
20:2	10.8±1.2	12.2±1.0	12.2±2.4
20:3	tr.	tr.	tr.
20:4(n−6)	49.8±1.0	48.0±1.5	44.6±1.6
20:5(n−3)	6.2±0.8	4.7±0.8	4.9±0.9
Saturated acid	23.5±2.5	24.9±1.8	26.9±1.8
Monoenoic acid	7.7±1.2	9.3±0.8	9.7±1.2
Polyenoic acid	68.8±2.3	65.8±2.2	63.4±2.8

The value is shown in mean±s.d.. tr., trace (<0.1%).

phosphatidylethanolamine in yolk granules was analyzed and the results are given in Table 4, 5 and 6, respectively. Fatty acids of triacylglycerol were mostly myristic (20:1) (12%), palmitic (16:0) (25%) and eicosamonoenoic (20:1) (12%) acids (Table 4). In the case of phosphatidylcholine and phosphatidylethanolamine, polyenoic fatty acids, such as arachidonic (20:4) and eicosapentaenoic (20:5) acids constituted more than 60% of the total fatty acid moieties, whereas those in triacylglycerol were approximately 40%. The predominant fatty acids in phosphatidylcholine were arachidonic (27%) and eicosapentaenoic (30%) acids (Table 5). Arachidonic acid was the major component of phosphatidylethanolamine (Table 6). Most of the lipids are evenly hydrolyzed during the incubation in acidic conditions.

DISCUSSION

Yolk granules occupy approximately 27% of the total volume of sea urchin eggs [1] and the number and mass of yolk granules do not change during embryogenesis [2, 12]. The current study reveals that ultrastructures within yolk granules change on the course of embryogenesis, and that the volume of yolk granules increases. The density of subparticles in yolk granules decreases gradually. These changes in yolk granules agree well with changes in protein composition of yolk granules observed so far [2, 9, 19, 20, 22]. Lysosome-like structures containing yolk granules were reported in the pluteus of *Arbacia punctulata* [2]. In *Hemicentrotus pulcherrimus*, yolk granules fused with lysosome-like structures were observed even in blastulae, and the occurrence of lysosome-like structures containing yolk granules increased as the embryonic development proceeds. Lysosomes presumably contribute to degradation of yolk granules at the later developmental stage by a pathway different from that for degradation of constituents within yolk granules. The fact that the volume of yolk granules increases during development seems to results from the changes in physicochemical properties of the membrane of yolk granules, which presumably have some relevance to hydrolysis of phospholipids.

Analyses of vitellogen and its proteolytic products by SDS-polyacrylamide gel electrophoresis and column chromatography revealed that the overall physical properties of the yolk protein

complex were well maintained after proteolytic processing. Electrophoretic analyses of proteins in 22S yolk glycoprotein complex [9] and yolk platelets [2] prepared by centrifugation of the sucrose density gradient have shown consistent results with that in the present work, although properties of yolk glycoproteins on ion exchange column chromatography have not been reported. Proteolytically processed vitellogen in blastulae, gastrulae or yolk granules incubated in acidic conditions can be resolved into its fragments by SDS-polyacrylamide gel electrophoresis in reducing conditions. These results imply that control of sulfhydril bondages play an important role for organization and dissociation of the yolk protein complexes. A glycoprotein complex mediating cell adhesion, toposome, and its relationship to vitellogen has been reported in sea urchin eggs and embryos [5, 7, 15]. Experiments using polyclonal and monoclonal toposome specific antibodies have indicated that glycoproteins originating from vitellogen are located in yolk granules and also in the intercellular area and surface area of the embryo. In this context, the mechanism controlling S-S bridges presumably has a key role in translocation of glycoproteins derived from vitellogen by proteolytic processing.

Besides sea urchins, degradation of vitellogen was reported to be activated in acidic conditions [6, 17]. Proteolysis of vitellogen in sea urchins is reported to be regulated by acidification [13]. The presence of several acid hydrolases, including acid phosphatase in yolk granules [18, 23] suggests that such catabolic enzymes can be used to generate fatty acids or carbohydrates from their stored macromolecular forms. Analysis of lipids in yolk granules incubated in acidic media indicates that catabolism of esterified fatty acids in yolk granules is generated by enzymes packaged in the same granules as in the case of proteolysis of yolk proteins.

Degradation of yolk granules during embryogenesis of the sea urchin presumably is divided into two processes. The one is hydrolysis of constituents packaged in yolk granule, which is dominant in the early development and is generated by the enzymes synthesized during oogenesis. The other is digestion of yolk granules by lysosomes, which occurs predominantly in the later developmental stage.

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